In the Specification

Please replace paragraphs [0001] through [0007] with the following:

Related Application

This is a §371 of International Application No. PCT/FR2003/003447, with an international filing date of November 21, 2003 (WO 2004/048581 A1, published June 10, 2004), which is based on French Patent Application No. 02/14600, filed November 21, 2002.

Field of the Invention

~~The~~This invention relates to ~~the area of~~ biology and, more especially, to the preparation of double-stranded oligonucleotides for use in a process of RNA interference (RNAi or ARNi).

Background

RNA interference, also designated "siRNA" or " RNAi" or also "co-suppression," has been demonstrated in plants, where it was observed that the introduction of a long double-stranded RNA corresponding to a gene induces the specific and efficacious repression of the target gene. The mechanism of this interference comprises the degradation of the double-stranded RNA into short duplexes of oligonucleotides of 20 to 22 nucleotides.

RNA interference has now been applied to mammals for specifically inhibiting genes for functional genetic applications. In fact, siRNAs permit the identification of the function of genes demonstrated by the sequencing of the human genome, either in models of cellular culture or in animal models, in particular, in the mouse. RNA interference is also useful in the therapeutic area for the treatment or prevention of cancers, infectious diseases and, more generally, diseases involving a heterologous or homologous mutated gene (S.M. Elbashir,, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber and T. Tuschl (2001a). Duplexes Of 21-Nucleotide RNAs Mediate RNA Interference in Cultured Mammalian Cells. Nature 411, pp. 494 - 498; S.M. Elbashir, J. Martinez,

A. Patkaniowska, W. Lendeckel and T. Tuschl (2001b). Functional Anatomy of siRNAs for Mediating Efficient RNAi in Drosophila Melanogaster Embryo Lysate. Embo J 20, 6877 - 6888).

The siRNAs are short sequences of double-stranded RNA that can be introduced in the form of synthetic oligonucleotides or in the form of plasmids permitting their transcription.

The use of plasmids has numerous advantages, in particular, for functional genetic applications. It permits the expression of double-stranded RNA in a stable manner in the cells and thus the easier inhibition of proteins with a long half-life. In fact, synthetic siRNAs have a half-life of 3 days in mammalian cells. It also permits long-term effects to be analyzed. On the other hand, it requires the establishing of lines expressing the construction in a stable manner, which has several disadvantages. In particular, it is necessary to compare stable lines with each other, which is generally difficult to interpret because the cellular lines drift. On the other hand, it is impossible to study the proteins indispensable for the cell, since their inhibition would block the proliferation of the cells and thus prevent ~~the establishing~~establishment of the stable line. It is therefore indispensable to be able to induce ~~at will~~ the expression of the siRNA at will.

Summary of the Invention

This invention relates to a method of expressing RNAi in cells, including introducing into eukaryotic cells a molecule of nucleic acid including sense and antisense sequences of RNAi placed under control of a promoter of single transcription, the sense and antisense sequences being separated by a sequence of DNA including a sequence for stopping transcription, wherein the DNA sequence is framed at each end thereof by a lox site, and placing Cre in contact with the lox sites to obtain by site-specific recombination elimination of the DNA sequence and the stop sequence of the transcription such that the sense and antisense sequences are no longer separated except by a remaining lox sequence and thereby permit transcription of the RNAi in its entirety with the

remaining lox sequence as a loop.

This invention also relates to a molecule of nucleic acid including sense and antisense sequences of RNAi placed under control of a promoter of single transcription, the sense and antisense sequences being separated by a sequence of DNA including a sequence for stopping transcription, wherein the DNA sequence is framed at each end thereof by a lox site.

This invention further relates to a cell or a cell line transfected by the molecule of nucleic acid.

This invention still further relates to a pharmaceutical composition including a therapeutically effective amount of an active substance of at least one molecule of nucleic acid and a compatible excipient.

Brief Description of the Drawings

Other advantages and characteristics of the invention will become apparent from the following examples that make reference to the attached drawings:

Fig. 1 shows the strategy for the expression of siRNAs in an inducible manner in accordance with the invention;

Figs. 2 and 3 show the induction of the activity of the RNAi by the CRE;

Fig. 4 shows the inhibition of the GFP marker by the RNAi;

Fig. 5 shows the inhibition of the GFP marker as a function of CRE during the course of transfection in two stages;

Fig. 6 shows the inhibition by RNAi of the GFP marker integrated in a cell line;

Fig. 7 shows the inhibition by RNAi of the endogenous gene p53 with establishment of stable cell lines; and

Fig. 8 shows the activity in vitro of RNAi.

Detailed Description

~~The~~This invention ~~has~~solves the ~~specific problem of palliating these~~ disadvantages noted above by making available a system for expressing an siRNA in a stable and inducible manner. This ~~problem~~ is ~~solved by the use of the~~achieved with a CRE-lox system for the expression of an siRNA in mammalian cells. The invention thus relates to a method of expressing RNAi in cells comprising:

~~- The introduction~~introducing into eukaryotic cells ~~of~~ a molecule of nucleic acid comprising the sequences sense and antisense of RNAi placed under the control of a promoter of single transcription, which sense and antisense sequences are separated by a sequence of DNA comprising a sequence for the stop of this transcription, which DNA sequence is framed at each of its ends by an lox site, and

~~- The~~ placing in contact ~~of~~ the lox sites with Cre ~~in order~~ to obtain by site-specific recombination the elimination of the DNA sequence and ~~of~~ the stop sequence of the transcription in such a manner ~~that~~ that these sense and antisense sequences are no longer separated except by the remaining lox sequence and thus permit the transcription of the RNAi in its entirety with the residual lox sequence as a loop.

~~According to a particular realization of the~~The method ~~of the invention said~~may include the molecule of nucleic acid ~~comprises~~comprising 5' toward 3', as shown in ~~figure~~Fig. 1, a transcription promoter compatible with ~~said~~the cells, the sense sequence of the RNAi, a first lox site, a DNA sequence comprising a transcription terminator, the second lox site and the antisense sequence of the RNAi.

Please replace paragraph [0010] with the following:

In fact, the invention permits the reliable analysis of human genes from a functional viewpoint in cells in culture or in animals and, in particular, in mice. In fact, there are systems

permitting the inducible expression of CRE in cells and in animals. In the mouse, the CRE can also be expressed in a tissue-specific manner permitting the inactivation of a gene specifically in these tissues.

Please replace paragraph [0012] with the following:

The DNA sequence separating the sense and antisense sequences of the RNAi and comprising the transcription terminator is advantageously a gene resistant to an antibiotic such as neomycin, thus, permitting in addition the selection of the transfected cells.

Please delete paragraphs [0015] through [0022].

Please replace paragraphs [0023] through [0024] with the following:

Example 1

The plasmid plox siRNA ~~comprises~~comprised a promoter Pol II controlling a gene resistant to an antibiotic, neomycin. The neomycin cassette ~~is~~was surrounded by lox sites. In a first phase, a promoter Pol III (H1) was inserted in the direction opposite to promoter Pol II. The promoter H1 introduced into the plasmid behind the second region loxp with the restriction enzymes Nhel and Xbal ~~is~~was obtained by PCR from the following primers:

5' CTAGCTAGCCCATGGAATTCGAACGCTGACGTC 3' Forward (SEQ ID NO. 1)

5' GCTCTAGAGTGGTCTCATACAGAACTTATAAGATTCCC 3' Reverse (SEQ ID NO. 2)

This plasmid ~~is~~was based on the plasmid pSUPER permitting the constitutive expression of siRNA and described by Brummelkamp et al.

Please replace paragraph [0027] with the following:

The psiRNA lox ~~is~~was obtained by inserting the entire DNA sequence of the siRNA directly after the promoter Hl at the level of the Xbal sites. The sense and antisense siRNAs ~~are~~were separated by a loop.

SiRNA:

5'CTAGTTTCCAAAAAAGCAAGCTGACCCTGAAGTTCATTCTCTTGAAATGAACTTCAGGGTCAGC TTGCGGGT 3' (SEQ ID NO. 7)

SiRNA complementary:

5'CTAGACCCGCAAGCTGACCCTGAAGTTCATTTCAAGAGAATGAACTTCAGGGTCAGCTTGCTTT TTTGGAAA 3' (SEQ ID NO. 8)

Please replace paragraphs [0028] through [0036] with the following:

Mammalian cells COS-7 were transfected with the polyfect (Qiagen) with 4 μg of expression vectors of the siRNA (plox siRNA, psiRNalox or plox) as indicated and a vector expressing the CRE recombinase or the corresponding empty vector (8 μg) as well as a vector for the expression of green fluorescent protein or GFP (500ng). Sixty hours after the transfection a Western blot was performed starting from the total extracts in using an antibody directed against the GFP (Santa cruz) or the cellular tubulin (Sigma) ~~in order~~ to evaluate the quantity of proteins used for this test (~~figure~~Fig. 2). Fibroblast cells (3T3) were transfected with 0.5 μg or 1 μg of the expression vector plox siRNA as indicated in ~~figure~~Fig. 3 and a vector expressing the CRE recombinase or the corresponding empty vector (8 μg) as well as a vector for the expression of GFP (500 ng). Sixty hours after the transfection a Western blot was performed starting from the total extracts using an antibody directed against the GFP (Santa cruz) or the cellular tubulin (Sigma) ~~in order~~ to evaluate the quantity of proteins used for this test (~~figure~~Fig. 3).

In the absence of CRE, the two constituent parts of the siRNA (sense and antisense strand are separated by the neomycin gene, that comprises a transcription stop sequence for Pol III. Under these conditions only the sense strand of the siRNA is transcribed and the siRNA is inactive: The target protein is normally expressed as shown in ~~figure~~Figs. 2 and ~~in figure~~ 3, third and fourth line. In the presence of CRE, the plasmid undergoes a process of recombination in the cell yielding a

product in which the neomycin sequence is eliminated and in which the two ½ siRNAs are only separated by the remaining lox sequence, in which there is no transcription stop sequence for Pol III. The siRNA is therefore transcribed in its entirety with the residual lox sequence that serves as a "loop". This siRNA is active and the target protein is inhibited (compare line 1 or 2 with line 3 or 4, ~~figures~~Figs. 2~~,~~ and 3).

~~The inhibition~~Inhibition is closely linked to the activity of the siRNA since in the presence of CRE the inhibition is only observed in the presence of the complete siRNA and not in the presence of the vector for the expression of the empty siRNA (line 1). Its activity is equivalent to that of an siRNA serving as a positive control, expressed in a constitutive manner (because the entirety of the sequence from which it is transcribed is placed in front of the neomycin gene).

On the other hand, the analysis by Northern shows the processing of the precursor and the synthesis of the siRNA induced by the CRE (~~figure~~Fig. 4). The total RNA of the COS-7 cells was extracted after 60 h of transfection then analyzed by northern blot with a probe marked at 32P directed against the antisense strand of the siRNAs produced:

5' CTTTCCAAAAAAGCAAGCTGACCCTGAAGTTCATG 3' (SEQ ID NO. 9)

~~Figure~~Fig. 4 shows that the inhibition is closely linked to the expression of the siRNA induced by the CRE.

Example 2

1) Methods

Plasmid constructions were realized in accordance with the method ~~presented in example~~of Example 1. The plox vector was constructed by inserting the promoter Pol III (H1) into the plasmid ploxNeo as an Nhel-Xba insert. The sequences corresponding to the sense and antisense siRNA

strands were introduced as synthetic oligonucleotides respectively using the restriction sites Xbal or BamHl and Kpn.

SiRNA sense:

5'CTAGCCCCGCAAGCTGACCCTGAAGTTCATT 3' (SEQ ID NO. 10)

SiRNA antisense:

5' GATCCATGAACTTCAGGGTCAGCTTGCTTTTGGTACCTAGACCC 3' (SEQ ID NO. 11)

This vector ~~will be~~was used in the subsequent examples.

Mammalian cells COS-7 were transfected in two stages. The first transfection was realized with 1 μg vectors for the expression of the siRNAs (plox siRNA, psiRNAlox or plox) and 2 μg of a vector expressing CRE recombinase or the corresponding empty vector. Twenty-four hours after this first transfection the cells were transfected with 500 ng of a vector for the expression of CMV-d2GFP (Clontech). A Western blot was realized in conformity with ~~example~~Example 1.

~~1)~~ ——Results

~~Figure~~Fig. 5 shows that GFP is undetectable during a transfection in two stages in the course of which the siRNA was able to form 24 hours before the transfection of the vector for the expression of GFP.

Please replace paragraphs [0038] through [0040] with the following:

~~Figure~~Fig. 6 shows the activity of siRNA on the expression of a GFP marker gene integrated into the genome of the cell line and inducible by doxycycline. The expression of the marker is observed in approximately 30% of the cells twenty-four hours after induction (~~figure~~Fig. 6A) and in 60% of the cells forty-eight hours after induction (~~figure~~Fig. 6B). Similar proportions are observed in the cells for the control of the transfection (positive RFP) transfected with the empty plox vector (~~figures~~Figs. 6A~~,~~ and 6B).

Independently of the expression of the CRE protein, no expression of the GFP target protein is observed in the cells transfected with the psiRNA plox vector expressing the siRNA in a constitutive manner. In the absence of CRE, among the cells transfected with the vector plox siRNA the proportion of positive GFP cells is approximately 30% one day after induction and approximately 65% two days after induction~~, but~~. However, this proportion is less than 5% in the presence of CRE.

Example 4

1) Methods

A plox siRNA expression vector directed against p53 (plox siRNA tp53) was constructed in accordance with the method presented in ~~example~~Example 2. The sense and antisense siRNA sequences used were the following:

SiRNA sense:

5'GCATGAACCGGAGGCCCATT 3' (SEQ ID NO. 12)

SiRNA antisense:

5'GATCCATGGGCCTCCGGTTCATGC 3' (SEQ ID NO. 13)

Please replace paragraph [0042] with the following:

~~2)~~ Results

~~Figure~~Fig. 7 shows three examples of clones transfected with the plox siRNA vector p53 presenting an inhibition of p53 dependent on CRE. No inhibition was observed in the clones ploxsiRNAp53 that were transferred with the empty vector pMC, as well as in the clones transfected in a stable manner with the empty vector plox. ~~Figure~~Fig. 7 shows the cell lines transfected in a stable manner in which a target endogenous gene is inhibited.

Please replace paragraphs [0044] through [0045] with the following:

Transgenic actin GFP mice of five to six weeks(Ikawa et al.) were anaesthetized with 300 μl of 0.05% xyalazine - 1.7% ketamine in NaCl 0.9%. After incision of the skin, 8 μg of DNA containing 3 μg of vectors for the expression of CRE and/or of the siRNAs and 2 μg of MCK-mlslacZ were injected into the *tibialis anterior* muscle (TA) with the aid of a 1 ml syringe provided with a 27 caliber needle. Caliper electrode plates (Q-biogen, France) ~~are~~were immediately applied on each side of the muscle and a series of eight electric pulses (2 Hz, 20 ms each) ~~is~~was delivered with a standard electroporator with a squarewave signal (ECM 830, Q-biogen). The electrical contact ~~is~~was ensured by the application of a conductive gel. Twelve days after the injection, the TA muscles ~~are~~were dissected and fixed on paraformaldehyde at 4% in PBS buffer (saline phosphate buffer) then incubated for two to three hours in 5-bromo-4-chloro-indol-β-galactoside 0.4 mg/ml, K3Fe (CN) 6 4 mM, K4Fe(CN) 6 4 mM and MgC12 2 mM, PBS at 37°C for the lacZ coloration. The positive LacZ regions ~~are~~were then dissected under the microscope. The images of fluorescence and in phase contrast ~~are~~were obtained with a Zeiss confocal microscope (LSM510, Zeiss).

Results

~~Figure~~Fig. 8 shows that the combination of the plasmid expressing CRE and of the plox siRNA GFP vector induces a marked reduction of the expression la GFP in the transfected fibers (the arrow indicates the positive LacZ nuclei). The expression of CRE in the presence of the plox control vector as well as the transfection of the ploxsiRNA vector in the absence of CRE do not affect the expression of GRP in the transfected fibers. ~~Figure~~Fig. 8 shows that the expression of the siRNA induced by CRE can reduce the expression of a gene in vivo.